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High-Level, Stable Expression of Blood Group Antigens in a Heterologous System

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The detection and identification of blood group antibodies in patients is crucial for successful allogeneic blood transfusions. Current methods are highly subjective and rely on red blood cells (RBCs), which simultaneously express many blood group antigens, have a short shelf-life, and carry potential biohazard risks. To overcome these problems, we have used the approach of expressing individual blood group antigen-bearing proteins in a heterologous system. We report here the high-level surface expression of type I (Knops), type II (Kell), and type III/multi-pass (Duffy) membrane proteins that carry blood group antigens in mouse erythroleukaemic (MEL) cells using a vector containing the β-globin locus control region. Importantly, the antigens expressed were detected specifically by a panel of patients' sera containing alloantibodies at sensitivities that are comparable to antigen-positive RBCs. Furthermore, in contrast to other mammalian expression systems, antigen expression was stable following freezing and thawing of the cell lines. Thus, this system has the potential both to replace the current use of RBCs by providing a one step method to detect and identify blood group antibodies and to allow the automation of antibody identification for the clinical laboratory. Am. J. Hematol. 63:114–124, 2000. © 2000 Wiley-Liss, Inc.

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INTRODUCTION

Over 250 blood group antigens on the surface of red blood cells (RBCs) have been defined by allo-antibodies. Of these, more than 200 have been classified into 25 blood group systems by the International Society of Blood Transfusion (ISBT) Working Party on Terminology for Red Cell Surface Antigens [1,2]. The genes encoding all but three of the blood group systems (Dombrock, Scianna, and P) have been cloned and sequenced, and the molecular basis of many individual antigens within the 25 systems has been determined [3,4]. Therefore, it is now possible to study individual blood group system antigens in isolation using expression systems.

Procedures currently used to detect and identify antibodies to blood group antigens are highly subjective and require specially trained personnel. Moreover, the techniques rely on RBCs, which must be carefully matched, have a short shelf-life, and may carry biohazard risks. Although antibody identification may be simple when a patient's serum contains only one specificity to a single blood group antigen, it can be complex when multiple antibodies are present. This is mainly due to the fact that the RBC membrane harbors many components that collectively carry multiple blood group antigens. The long-standing problems associated with the use of RBCs for detection and identification of antibodies could be overcome if it were possible to express a single RBC membrane protein that expresses defined antigens in a heterologous system. For this approach to be successful, the expression levels of the protein carrying the blood group antigen must be high enough to allow detection by low-titer alloantibodies with a level of specificity and sensi-

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tivity that equals conventional hemagglutination methodologies. Such an expression system would allow for the development of a one-step method to detect and identify blood group antibodies and could potentially simplify the process of antibody identification by allowing the development of an objective, automated test system.

The Kell antigens are carried on a type II single-pass membrane glycoprotein that has homology with neutral zinc dependent endopeptidases [5,6]. It was recently shown that Kell has indeed enzymatic activity [7]. Twenty-four highly conformation-dependent antigens have been associated with the Kell blood group system, thirteen of which are carried on the wild-type Kell protein and are therefore referred to as high-incidence antigens. The molecular bases of most Kell antigens have been determined and are associated with point mutations encoding different amino acids [6]. Previous reports have shown that Kell protein can be expressed on the surface of K562, COS-1, and insect cells where it can be readily detected by monoclonal antibodies (mabs) [6,8]. However, although there was noticeable binding of human polyclonal alloantibodies to several Kell antigens in transfected cells, a high level of background binding with K562 and COS cells was observed [8]. Therefore, these cell lines are not suitable for the specific purpose of antibody detection since the expression system needs to have a low level of background reactivity with antibodies.

The Duffy blood group system consists of six antigens, four of which are of high incidence. The Duffy protein is a promiscuous chemokine receptor [9–13] that is predicted to be a multipass RBC protein [13,14] and is the receptor for the human malarial parasite *Plasmodium vivax* [15] as well as a related simian parasite *Plasmodium knowlesi* [16]. The Duffy protein has been expressed in K562 cells [12,17], human erythroleukaemia cells (HEL) [18], and in a number of non-erythroid cell lines [13,17–19] as detected by mabs. The two antithetical antigens of Duffy, namely Fy^a and Fy^b, were demonstrated by surface expression in COS cells by transient transfection using strongly reactive commercial anti-Fy^a and anti-Fy^b, respectively [20]. However, for routine use in antibody identification in the clinical laboratory, a stable cell line that reproducibly expresses high levels of antigen is a prerequisite.

Antigens in the Knops blood group system are carried on complement receptor 1 (CR1) [21,22] which is a single-pass type I membrane protein and is the receptor for C3b/C4b [23,24]. CR1 has been implicated in rosette formation following invasion of RBCs by *Plasmodium falciparum* [25]. The five antigens of this blood group system have not been mapped. In contrast to alloantibodies in the Kell and Duffy blood group systems, alloantibodies in the Knops system are considered to be clinically insignificant. CR1 has been transfected and expressed in COS and L cells as the native membrane attached protein [26] and as an engineered secreted form [27]. However, the protein expression levels were not stable following freezing and thawing of CR1-expressing cells (our unpublished observation).

Murine erythroleukaemic (MEL) cells [28] have been extensively used as an erythroid tissue culture model system. MEL cells are Friend virus-transformed erythroid progenitor cells arrested at the proerythroblast stage of differentiation. Upon treatment with various chemical agents, including dimethyl sulfoxide (DMSO), these cells are induced to undergo terminal differentiation that closely mimics the analogous process in vivo [29,30]. The human β-globin locus control region (LCR) sequences [31] confer integration-independent, high-level expression on stably transfected genes which are linked in cis in MEL cells [32,33]. By using expression constructs that contain the LCR, a number of non-erythroid proteins have been successfully expressed on the surface of MEL cells [34–38] We therefore tested whether the erythroid-specific LCR/MEL expression system could be used to direct stable expression of blood group antigens at levels comparable to those on RBCs and in the correct conformation so that they can be used for antibody detection.

Using the LCR/MEL system, we describe here high-level surface expression of proteins carrying Knops, Kell, and Duffy blood group antigens as model systems for type I, II, and III proteins, respectively. The level of expression obtained with each protein was comparable to that on antigen-positive RBCs and was stable on freezing and thawing. Moreover, recombinant antigens expressed on the transfected cells were detected specifically by alloantibodies from donor or patient sera. Our results imply that this system has the potential for use in antibody detection and identification in the clinical setting, which is crucial for successful allogeneic blood transfusions.

MATERIALS AND METHODS

Materials

RBCs with known antigen types were obtained either from local blood donors, a commercial panel (Gamma Biologicals, Inc., Houston, TX), or from frozen storage. Mab anti-K14 (6–22) [39] and anti-Fy6 (NYBC-BG6, clone K6) [40] were kindly supplied by Pablo Rubinstein (New York Blood Center). The mab anti-Fy3 (CRC-512-1) was kindly provided by Makato Uchikawa (Japanese Red Cross, Tokyo, Japan). Mab anti-CR1 (DAKO-CD35, To5) was purchased from Dako Corporation (Carpinteria, CA). Commercial antibodies were from Gamma Biologicals, Inc. (Houston, TX) and Ortho Diagnostic Systems, Inc. (Raritan, NJ). Sera containing alloantibodies with specificities identified by the Immu-

nohematology Laboratory at the New York Blood Center were obtained from blood donors or patients. Sera used included the following Kell specificities: anti-k (BK, GUE), anti-Kp^a (JH), anti-Kp^b (RAU, 303255), anti-Ku (JM, KH), anti-Js^b (NM, CHI), anti-K11 (COT), anti-K12 (MS), anti-K13 (AS), anti-K14 (CS), anti-K18 (MAR), anti-K19 (MON), anti-K22 (IN). Donor sera containing anti-Fya (for the identification numbers of specific antibodies see Fig. 2 legend and Table II) or anti-Fy^b (107840) and one patient serum (WED) with anti-Fy^b specificity were used. Patient sera with anti-Knops blood group system specificities tested included anti-Sl^a (65-97, 262-97; 411-97, 961-97), anti-Kn^a (1217-96, 374-97, 777-97), anti-Kn^a/Yk^a (332-97, 649-97, 716-97), and anti-Kn^a/Sl^a (765-97).

Construction of the Expression Vectors

A 2.2 kb cDNA containing the entire coding region of the wild-type Kell protein [41] was subcloned into pBCSK vector (Strategene) at the *Eco*RI polylinker site. This was then isolated as a *BamH*I-SalI fragment and inserted into the β-globin LCR expression vector pEV [37] using *Bgl*II and *Sal*I cloning sites.

The full-length1.4 kb cDNA encoding the Fy^a antigen [19,20] was subcloned into the *Sma*I site of pBluescript-SK vector (Strategene) and then released by digestion with *BamH*I and *Sal*I and inserted into the *Bgl*II/*Sal*I sites of pEV. The Fy^b full-length cDNA was isolated as a *BamH*I-*Not*I fragment from plasmid pcDNA1 (Invitrogen) [12] and subcloned into the *BglII*/*Not*I sites of pEV. The unspliced forms of the genes [42] were used.

The CR1 cDNA was kindly provided by Lloyd Klickstein (Division of Rheumatology, Immunology & Allergy, Brigham & Women's Hospital). A 6.9 kb *Sal*I/*Not*I fragment containing the entire CR1 cDNA coding sequence was isolated from piABCD [26] and ligated into the *Sal*I/*Not*I sites of pEV.

Tissue Culture and Cell Transfection

MEL-C88 cells were maintained and transfected by electroporation as previously described [29]. Briefly, 50 μg of the pEV3 vector alone or containing Kell or Duffy cDNAs were linearized by digestion with *Pvu*I, a unique site present in the ampicillin resistance gene of the plasmid backbone. The CR1 expression vector was tranfected as a supercoiled plasmid. In each case, 10^7 cells were transfected with the appropriate expression constructs by electroporation (250 V, 960 μF using the Bio-Rad Gene-Pulser) [29]. Directly after transfection, cells were diluted in culture medium to 10^5 cells per ml and aliquots of 1 ml were transferred to each well of a 24-well plate. Forty-eight hours after transfection, G418 was added at a final concentration of 800 μg/mL to select for stable transfectants. Individual G418-resistant clones were picked 10–14 days after the addition of selection medium to obtain stable cell lines.

Detection of Surface Expression by Flow Cytometry

Stable transfected MEL cell clones were stimulated to undergo terminal erythroid differentiation for 4 days in the presence of 2% (v/v) dimethyl sulfoxide (DMSO) [29] in order to induce maximum expression of the pEV-based transgenes [34]. Parental MEL cells which were not transfected and/or MEL cells transfected with "empty" pEV-3 vector were used as negative controls, and RBCs of the appropriate phenotype were used as positive controls. Briefly, 10^6 MEL cells or RBCs (10 μl of 0.5% suspension) were washed once with phosphate-buffered saline at pH 7.3 (PBS) containing 0.5% bovine serum albumin (BSA). The cells were incubated for 1 hr at 37°C with the appropriate murine mabs or human allo-antibodies with a final dilution of 1:2 in PBS/0.5% BSA. After three washes, the cells were incubated with fluorescein-conjugated anti-mouse IgG (made in horse) or anti-human IgG (made in goat) (H+L) (Vector Laboratories, Burlingame, CA) at 50-fold dilution for 30 min at 4°C. Following two washes in PBS/0.5% BSA, 10 μg of propidium iodide (PI) was added to 0.5 ml of each cell suspension just before analysis on a FACSCalibur flow cytometer (Becton Dickinson, CA). Only PI-negative cells (over 80% of the cell population) representing the live cells were selected and are shown in the histograms. Mean fluorescence intensity was used as a measure of antibody binding.

Adsorption of Allo-antibodies

One volume of serum or plasma containing the allo-antibody of interest was incubated with an equal volume of washed cells (4-day 2% DMSO-induced transfected or untransfected cells, or antigen-positive or antigen-negative RBCs). Typically, 100 μl of packed cell volume of RBCs or MEL cell clones (equivalent to 10^8 cells) were used. Following incubation for 1 hr at 37°C, the mixture was separated by centrifugation. The absorbed fluid was then removed, serially diluted, and tested against antigen-positive RBCs by the indirect anti-human globulin test by hemagglutination using standard tube techniques. The titration score of the antibody was used to indicate the strength and titer and was calculated by combining the individual scores of the antibody reactivity at each dilution point.

Stability on Freezing and Thawing

Stable transfected MEL cell clones that had been frozen in media containing 10% DMSO [29] were re-established in culture and induced to undergo erythroid differentiation. They were then refrozen (at −80°C or liquid nitrogen) as induced cells in the media containing

10% DMSO. A week later, following thawing, they were immediately washed once with PBS containing 0.5% BSA and tested for expression of blood group antigens by flow cytometry and for their ability to adsorb specific antibodies as before.

RESULTS

Expression of Kell Antigens by Flow Cytometry

Of 39 stable MEL clones that had been transfected with wild-type Kell in the pEV expression vector, 38 expressed Kell protein to varying degrees as detected by mab anti-K14 by flow cytometric analysis (data not shown). The highest expressing clone, MEL.KEL.24, was selected for further analysis. Throughout the analyses, an MEL cell clone that had been transfected with "empty" vector was used as negative control and the relative shift in mean fluorescence intensity was used as a measure of antibody binding. The expression of the K14 antigen on MEL.KEL.24 cells was similar to that on antigen-positive RBCs (Fig. 1, monoclonal anti-K14). In addition, commercial reagents [anti-k ($n = 2$), anti-Kp^b ($n = 2$)] also detected the corresponding antigens on the MEL.KEL.24 cells (data not shown). These results indicate that at least three conformation-dependent Kell blood group system antigens (k, Kp^b, K14) were present on the MEL.KEL.24 cells.

We then tested whether MEL.KEL.24 cells could be detected by patient or donor sera containing alloantibodies to Kell system antigens. Serum or plasma from patients or donors containing anti-k ($n = 2$), anti-Kp^b ($n = 2$), and anti-Js^b ($n = 2$) were analyzed for their ability to detect the corresponding antigens on MEL.KEL.24 cells (see Fig. 1 for representative histograms). Not only did these antibodies detect these high-incidence antigens on MEL.KEL.24 cells, but the level of sensitivity of detection was also comparable to that of antigen-positive RBCs (Fig. 1, compare the RBC panels with the MEL.KEL.24 panels). The expected absence of expression of the antithetical low-incidence antigens was confirmed by the lack of staining of the cells with anti-K, anti-Kp^a, and anti-Js^a allo-antibodies (data not shown), thus showing the specific nature of antigen expression.

A number of patient sera or plasma containing alloantibodies to other high-incidence antigens associated with RBCs that express the wild-type Kell protein were also tested. These included examples of (allo)anti-Ku, anti-K11, anti-K12, anti-K13, anti-K14, anti-K18, anti-K19, and anti-K22 (Fig. 1). Each antibody detected the corresponding antigen on MEL.KEL.24 cells at levels equivalent to antigen-positive RBCs. Together, these data demonstrate that the Kell protein on MEL.KEL.24 cells is expressed at levels comparable to those on RBCs and that the protein is present in its wild-type conformation on the cell surface as demonstrated by the expression of multiple Kell antigens. More importantly, anti-Kell alloantibodies from donor or patient sera react specifically with MEL.KEL.24 cells and give a low background with the parental MEL cells.

Adsorption of Kell Antibodies

In the clinical laboratory, a specific antibody may be removed from serum by adsorption as part of the antibody detection and identification procedure. Red cells that express the specific antigen are incubated with the test serum, and if there is specific adsorption by the cells, the antibody(ies) will be absorbed and the serum will show a decline in antibody reactivity. In order to determine whether the MEL.KEL.24 cells can be used to adsorb antibodies and thereby replace RBCs in the clinical laboratory, we tested several clinically significant alloantibodies to high-incidence Kell antigens, namely, anti-k ($n = 2$), anti-Kp^b ($n = 2$), anti-Js^b ($n = 2$), and anti-Ku ($n = 2$). Untransfected MEL cells were used as a negative control for nonspecific adsorption. As shown in Table I, MEL.KEL.24 completely and specifically adsorbed all high-incidence antibodies tested except for one anti-k (BK) serum. This anti-k gave similar results with antigen-positive RBCs after one adsorption (the titration score was reduced from over 60 to 9), thus indicating that the MEL.KEL.24 cells behave similarly to RBCs in their adsorption ability. Two adsorptions of this anti-k with MEL.KEL.24 cells completely removed the alloantibody from the serum. In addition, when checked against a patient serum containing a low-incidence alloantibody (anti-Kp^a), no adsorption was obtained with MEL.KEL.24 cells (Table I), indicating that the adsorption ability of these cells is specific for high-incidence antibodies. Collectively, these data show the potential of the transfected cell line in the clinical laboratory for adsorption studies.

Stability Tests

In order to test the stability of Kell antigen expression on MEL.KEL.24, the cells, following stimulation to undergo terminal differentiation, were frozen in liquid nitrogen and then tested by adsorption and flow cytometry. The MEL.KEL.24 cells completely adsorbed anti-k (GUE; score 34), anti-Js^b (NM; score 49), and anti-Ku (JM; score 56). Moreover, expression, as measured by flow cytometry with anti-k, anti-Kp^b, and anti-K14, was comparable to that obtained with cells that had been kept in culture continuously (data not shown). Similar results were obtained when the cells were frozen at −80°C (data not shown). These data shows that, in contrast to other Kell transfected cell lines such as K562 and COS cells (our unpublished observations), the expression of Kell antigens on MEL.KEL.24 cells is stable upon freezing and thawing.

Expression of Duffy Antigens by Flow Cytometry

Expression of the Duffy antigens in the transfected MEL clones was determined by flow cytometry using

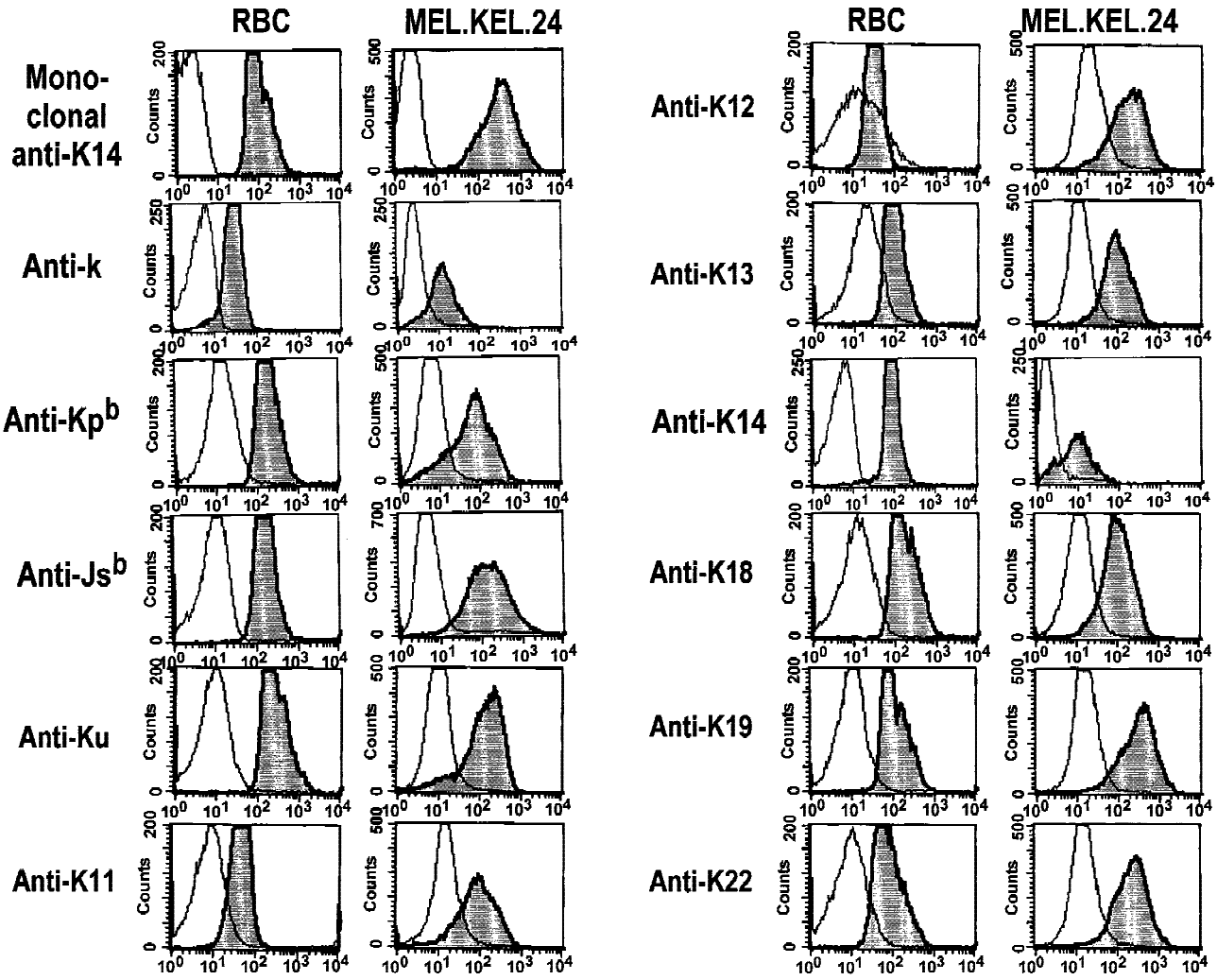

Fig. 1. Flow-cytometric analysis of MEL.KEL.24 cells. The ability of MEL.KEL.24 cells to express wild-type Kell associated antigens and to detect human allo-antibodies from patient sera was tested by flow cytometry. For every antibody tested, parallel staining and flow-cytometric analysis were performed with RBCs and the transfectants. RBCs with the common Kell phenotype [K−k+, Kp(a−b+), Js(a−b+), Ku+, K:11,12,13,14,18,19,22] were used as the positive control while antigen-negative RBCs were used as the negative control in the "RBC" testing. In the "MEL.KEL.24" histograms, MEL cells that had been transfected with "empty" vector were used as the negative control. The results are depicted as overlays of open histograms to represent negative control cells/RBCs and shaded histograms, representing positive control RBCs or MEL.KEL.24 cells. Mean fluorescence intensity (as a measure of antibody binding) in log scale is on the x axis, and the relative number of cells is represented on the y axis. All antibodies tested, except for the monoclonal anti-K14, were from patient's sera. MEL.KEL.24 cells can be used to detect alloantibodies with all the tested specificities. Moreover, the level of sensitivity of detection, as demonstrated by the relative shift in fluorescence intensity, is comparable to that of antigen-positive RBCs. Patient sera used were as follows: anti-k (BK), anti-Kp^b (RAU), anti-Js^b (NM), anti-Ku (JM), anti-K11 (COT), anti-K12 (MS), anti-K13 (AS), anti-K14 (CS), anti-K18 (MAR), anti-K19 (MON), anti-K22 (IN).

mab anti-Fy6 (data not shown) which recognizes an epitope in the N-terminal extracellular region of the Duffy protein [43,44]. The highest Fy^a-expressing cell line, MEL.FYA.21 (chosen from 63 clones), and the highest Fy^b-expressing cell line, MEL.FYB.37 (chosen from 58 clones), were selected for further analysis. Expression of Duffy protein was also demonstrated by mab anti-Fy3 (Fig. 2) whose specificity lies in the third extracellular loop of the Duffy protein [17]. These results show that both MEL.FYA.21 (Fig. 2, middle column) and MEL.FYB.37 (Fig. 2, right-hand column) transfectants express epitopes corresponding to amino- and carboxyl-terminal regions of the Duffy protein, indicating that the proteins are expressed in a correct orientation on the surface of the transfected cells. Furthermore, commercial anti-Fy^a ($n = 3$) and anti-Fy^b ($n = 2$) reacted specifically with the two transfectants at levels equal to RBCs (data not shown).

The cell lines were then tested for their ability to detect anti-Fy^a or anti-Fy^b in patient and donor sera. Since the

TABLE I. Antibody Adsorption With MEL.KEL.24 Cells*

Alloantibody		Titration score before adsorption	Titration score after adsorption with MEL	Titration score after adsorption with MEL.KEL.24
Anti-k	(GUE)	34	34	0
	(BK)	60[b]	67[b]	18[c]
Anti-Kp[b]	(RAU)[a]	33	31	0
	(303255)	26	26	0
Anti-Ku	(JM)	56	56	0
	(KH)	62	54	0
Anti-Js[b]	(NM)	49	49	0
	(CHI)	26	26	0
Anti-Kp[a]	(JH)	26	23	25

*Alloantibodies to Kell antigens were used to test the ability of MEL.KEL.24 cells for their adsorptive capacity as described in Materials and Methods. Parental MEL cells were used as a negative control. Identification numbers of commercial sera or donor sera, and initials of sera from patients are indicated next to each antibody specificity.

[a]Anti-Kp[b] (RAU) serum also contained anti-K.

[b]The titration score was greater than the number listed since the antibody score was still high at the highest dilution commonly used to calculate the titration score.

[c]When antigen-positive RBCs were used to adsorb this anti-k (BK), the titration score was reduced from 60 to 9, indicating that the adsorption ability of MEL.KEL.24 cells is similar to that of RBCs.

Fy[a] antigen is more immunogenic and less common in the population than the Fy[b] antigen, there are a greater number of anti-Fy[a] allo-antibodies available than anti-Fy[b]. Eleven examples of anti-Fy[a] and two examples of anti-Fy[b] were used to test MEL.FYA.21 and MEL.FYB.37 cells by flow cytometry. Representative examples are shown in Fig. 2. Anti-Fy[a] reacted with MEL.FYA.21 cells but not with MEL.FYB.37 cells, while anti-Fy[b] specifically detected the MEL.FYB.37 clone. Since the Fy[a] and Fy[b] transfected genes differ by a single point mutation (encoding G44D), these data confirm that a single amino acid change is sufficient to define the antithetical antigens, Fy[a] or Fy[b]. In addition, the transfectants express Duffy antigens at levels comparable to antigen-positive RBCs and can be detected specifically by anti-Fy[a] and anti-Fy[b] allo-antibodies from donor or patient sera.

Adsorption of Duffy Antibodies

The ability of MEL.FYA.21 and MEL.FYB.37 cells to specifically adsorb sera with anti-Fy[a] and anti-Fy[b] was examined. Anti-Fy[a] ($n = 3$) were adsorbed by MEL.FYA.21 cells but not by MEL.FYB.37 cells, while MEL.FYB.37 cells specifically adsorbed anti-Fy[b] ($n = 3$) (Table II). Antibodies that were not completely removed by one adsorption with the transfectant expressing the corresponding antigen, gave similar results using antigen-positive RBCs. These results show that both MEL.FYA.21 and MEL.FYB.37 cells are capable of specifically adsorbing anti-Fy[a] and anti-Fy[b] to a similar degree as antigen-positive red cells.

Expression Analysis of CR1 by Flow Cytometry

The entire CR1 cDNA [26] was transfected into the MEL/C88 cells, and, following establishment of stable clones ($n = 60$), surface expression was demonstrated in 29 clones by flow cytometry using a mab anti-CR1 (data not shown). The highest CR1-expressing clone, MEL.KN.39, showed a higher level of protein expression than the test RBCs (Fig. 3). Alloanti-Knops are characteristically weakly reactive and can be difficult to identify serologically. Using flow cytometry as the method of detection, it was not possible to demonstrate the expression of Knops antigens on either RBCs or on MEL.KN.39 using a panel of nine anti-Knops system alloantibodies (data not shown).

Adsorption of Knops Antibodies

MEL.KN.39 cells were used to adsorb several sera containing anti-Knops. As shown in Table III, while adsorption with parental (untransfected) MEL cells weakened the reactivity in all eleven sera (see Discussion), adsorption with MEL.KN.39 cells led to a more effective removal of the anti-Knops. Antibodies that were partially adsorbed with MEL.KN.39 cells gave similar results with RBCs (in Table III, samples marked with asterisks). These data indicate that MEL.KN.39 cells specifically adsorb Knops system antibodies and that their adsorption ability parallels that of antigen-positive RBCs.

DISCUSSION

We have obtained, in the LCR/MEL expression system, high-level expression of three types of RBC membrane proteins, namely, Kell (single-pass, type II), Duffy (multi-pass, type III), and CR1 (single-pass, type I). Antigens in these blood group systems were expressed in MEL cells at levels that are equivalent to antigen-positive RBCs, indicating that the proteins reflect the same conformation as in the RBC membrane milieu. For practical clinical applications, it is essential to have high-level expression of the proteins (and, thus, the associated antigens) to allow their use for the detection of alloantibodies in serum from a donor or patient. While high-level expression of blood group antigens has been achieved following transient transfection of the genes into mammalian cell lines [20], or injection of RNA into *Xenopus* oocytes [45], these systems have the limitation of expressing the proteins transiently. In contrast, the LCR/MEL system allows stable expression of heterologous proteins which is critical if an expression system is to have utility in the clinical laboratory. Although stable expression of other recombinant blood group systems has been achieved in the past [46], we have found that antigen expression in several expression systems deteriorated on freezing and thawing (our unpublished observa-

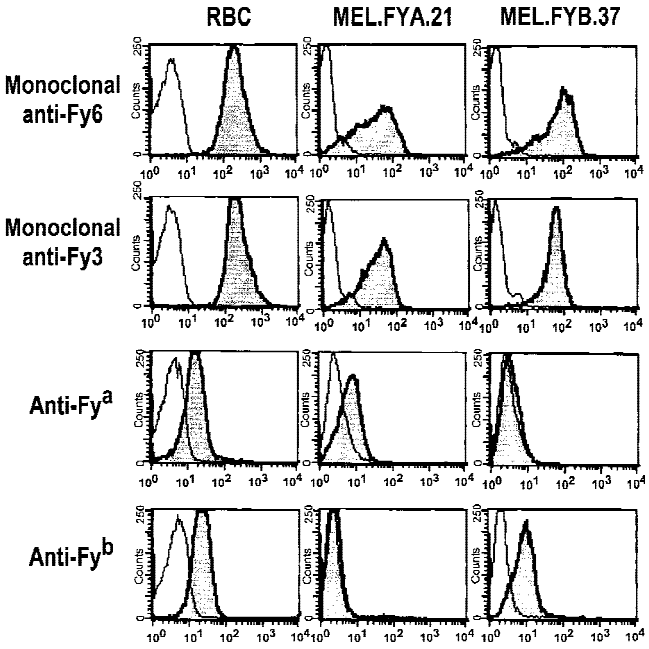

Fig. 2. Flow-cytometric analysis of MEL.FYA.21 and MEL. FYB.37 cells. MEL.FYA.21 cells (middle column) and MEL. FYB.37 cells (right-hand column) were tested in parallel with antigen-positive RBCs (left-hand column) for antigen expression and for their ability to detect human alloantibodies by flow cytometry. Antigen-negative RBCs with Fy(a−b−) phenotype and MEL cells transfected with "empty" vector cells were used as negative controls for antibody staining of RBC and transfectants, respectively. The results are shown as histogram overlaysas in Fig. 1. Mab anti-Fy6 and mab anti-Fy3 demonstrated the presence of the corresponding antigens on the transfectants. Serum from a donor with anti-Fya (#2788909) detected expression of Fya antigen in MEL.FYA.21 cells at a level comparable to Fy(a+b−) RBCs but did not detect MEL.FYB.37 cells. Patient serum (WED) containing anti-Fy^b reacted with MEL.FYB.37 cells and Fy(a−b+) RBCs to a similar degree and did not react with MEL.FYA.21 cells.

tions) but that of MEL transfected cells did not. Furthermore, the cells can be air-dried (or fixed using mild conditions) onto plastic without loss of antigen expression (our preliminary results). Thus, it is possible to prepare the transfected cells in bulk and either to freeze them in aliquots or attach them to plastic surfaces for future use. Since MEL cells are non-adherent and fast-growing (doubling time of 10–16 hr), they are straightforward to culture in large-scale fermentors. Through the use of the LCR/MEL system, stable clones expressing high levels of the recombinant protein can be obtained in a relatively short time (about 3 weeks from the time of transfection). This is unlike the baculovirus/insect system which also offers stable high-yield expression of introduced genes, but has the disadvantages of requiring extra steps in the preparation of virus and of controls for infection and lysis.

TABLE II. Antibody Adsorption With MEL.FYA.21 and MEL.FYB.37 Cells*

Alloantibody	Titration score before adsorption	Titration score after adsorption with MEL.FYA.21	Titration score after adsorption with MEL.FYB.37	Titration score after adsorption with Ag+ RBCs
Anti-Fy^a				
(6769292)	53	0	38	ND
(1416553)	38	16[a]	35	19[a]
(2943986)	67[b]	6	53[b]	ND
Anti-Fy^b				
(FYB35A1)[c]	47	47	0	ND
(FYB37D)[c]	32	35	6[a]	9[a]
(WED)	33	32	19[a]	14[a]

*Sera containing anti-Fy^a or anti-Fy^b were used to demonstrate the adsorption ability of MEL.FYA.21 and MEL.FYB.37 cells. To control for specificity of adsorption of anti-Fy^a, MEL.FYB.37 cells (that do not express the Fy^a antigen as shown by flow cytometry in Fig. 2) were used as the negative control. Conversely, MEL.FYA.21 cells (which do not express the Fy^b antigen; see Fig. 2) were used as negative control for anti-Fy^b.
[a]Similar partial adsorptions were observed when using Fy(a+b+) RBCs, indicating that the cell lines had similar adsorption capacities to RBCs.
[b]Defined as in legend to Table I.
[c]Commercial antibodies.

It is possible that certain blood group antigens may require erythroid-specific interacting factors/proteins that specifically influence their expression at the cell surface in a heterologous cell line. Such factors will be absent or modified in non-erythroid expression systems. The human erythroid K562 cells have been used extensively for the transfection and expression of several blood group antigens [8,12,47,48]. However, they express many endogenous blood group antigens which can be detected by alloantibodies [49,50]. This can result in a high background of antibody binding, making them unsuitable for antibody detection purposes. In contrast, MEL cells, which are also erythroid but of mouse origin, do not cross-react with a large number of human antibodies (this paper and our unpublished data) [51] except those belonging to the Knops blood group system (see below). Thus, the MEL expression cell line gives a low background, thereby allowing for a high signal to noise ratio which is essential if transfected cells are to be used for the detection of antibodies. Moreover, as they do not elicit anti-species reactions in mice, MEL cells expressing blood group antigens can be used as immunogens for the production of monoclonal antibodies [52]. As part of the current antibody detection and identification process in the clinical laboratory, adsorption may be used to separate mixtures of antibodies present in a serum. Moreover, adsorption is used to remove high-incidence alloantibodies or auto-antibodies from a serum in order to detect underlying antibodies that may be potentially of clinical significance. The ability of the MEL.KEL.24 cells to specifically and efficiently adsorb clinically significant alloantibodies to high-incidence antigens,

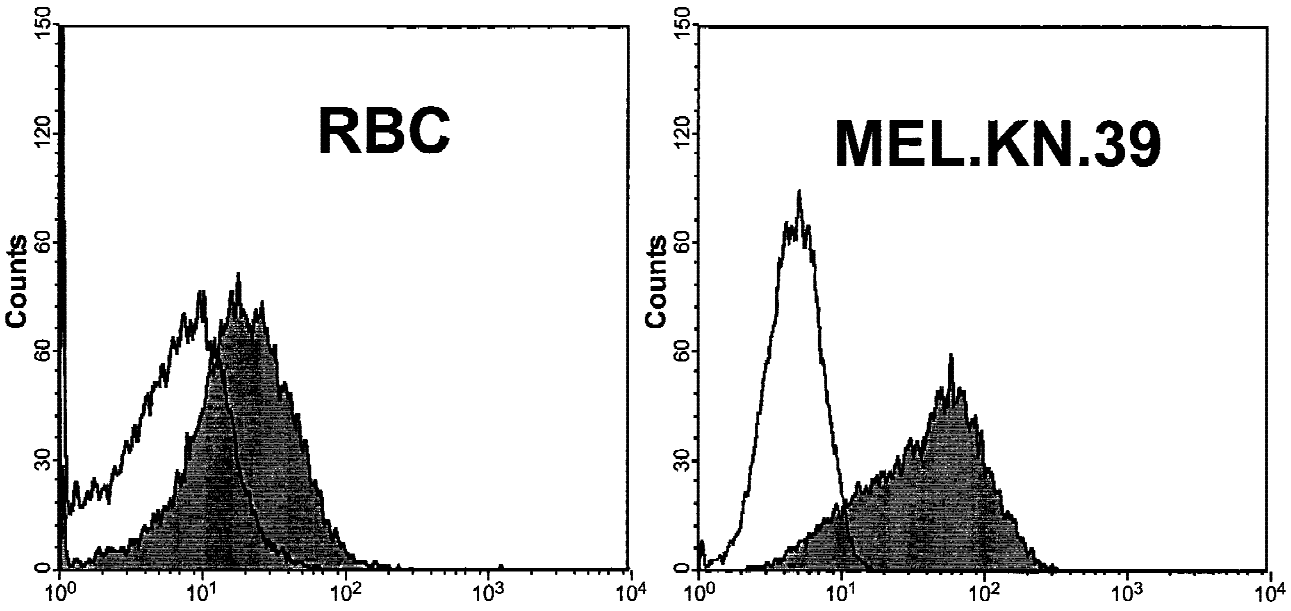

Fig. 3. Immunofluorescence analysis of CR1 expression on MEL.KN.39 cells. Murine mab anti-CR1 and negative control antibody were used to measure the expression level of CR1 on normal RBCs (left histogram). Using the same anti-CR1 mab, parental MEL cells were non-reactive while MEL.KN.39 cells expressed the protein at levels higher than those on RBCs (right histogram).

TABLE III. Antibody Adsorption With MEL.KN.39 Cells*

Alloantibody	Titration score before adsorption	Titration score after adsorption with		
		MEL	MEL.KN.39	Ag+ RBCs
Anti-S1[a]				
(961-97)	7	5	0	ND
(65-97)	10	6	0	ND
(411-97)	2	2	0	ND
(262-97)	11	8	8[a]	10[a]
Anti-Kn[a]				
(374-97)	11	5	0	ND
(777-97)	6	3	1	ND
(1217-96)	19	14	14[a]	14[a]
Anti-Kn[a]/Yk[a]				
(649-97)	5	3	0	ND
(332-97)	11	4	0	ND
(716-97)	6	2	2[a]	8[a]
Anti-Kn[a]/S1[a]				
(765-97)	7	3	0	ND

*The adsorption ability of MEL.KN.39 cells to specifically remove anti-Knops alloantibodies from patient sera was tested. Since anti-Knops antibodies are characteristically weakly reactive, scores of 4 and below for a given antibody dilution point are also included when calculating the titration scores.

[a]Adsorption with antigen-positive RBCs to demonstrate similar partial adsorptions.

namely anti-k, anti-Kp[b], anti-Js[b], and anti-Ku, provides a means whereby these antibodies, when present in patient sera, can be identified directly. This has a tremendous value in the clinical laboratory since it allows for a one-step method to detect and identify these blood group antibodies. While the adsorption ability of the Duffy and CR1 transfected cells was not as efficient as the Kell transfectants, it was similar to that obtained using antigen-positive RBCs. The results imply that the recombinant proteins have the potential to completely adsorb the antibodies if an increased ratio of cells to serum is used or a second adsorption is performed, as is usually undertaken in such cases in the clinical setting when using RBCs.

In contrast to antibodies to Kell and Duffy blood group system antigens, antibodies to the Knops blood group system antigens (Kn[a], McC[a], Sl[a], Yk[a]) are characteristically weakly reactive, hard to identify, and generally considered clinically insignificant [53]. It would be useful, therefore, to remove such clinically insignificant antibodies to prevent their interference in pretransfusion compatibility testing. By using an expressing cell line such as MEL.KN.39 cells, antibodies to the Knops blood group system antigens can be absorbed from a patient's serum. This approach can have practical utility in the clinical laboratory since it will allow routine crossmatching and transfusion of antigen-positive blood without the need for identification of anti-Knops antibodies. Others have shown that inhibition of Knops system antibodies occurs if a highly concentrated preparation of soluble CR1 is used [54]. Interestingly, the parental MEL cells caused a weakened reactivity of the Knops system antibodies in our adsorption studies. Since these cells did

not noticeably reduce the reactivity of antibodies to Kell and Duffy system antigens, it is unlikely that dilution or change in physical conditions (e.g., pH, ionic strength) of the test media could account for this unexpected weakening. It is possible that MEL cells have endogenous mouse CR1 expressing antigens that cross-react with some of the human Knops system antibodies or with a component, unrelated to CR1, that is present on the membrane of MEL cells. Nevertheless, when MEL.KN.39 cells that expressed CR1 were used, a more effective adsorption of Knops antibodies (anti-Kn^a, anti-Sl^a, and anti-Yk^a) was achieved. Together these results indicate that transfected MEL cells expressing individual blood group antigens can potentially be used to replace RBCs in the clinical setting to adsorb specific antibodies as part of antibody identification and detection or before compatibility testing.

In addition to their potential as clinical reagents, the high-level expression obtained in the LCR/MEL system has paved the way for studying biological functions of blood group system proteins. Kell was recently shown to process endothelin 3 and to some extent endothelin 1 [7]. MEL.KEL.24 cells may be useful in assaying other potential substrates of Kell. Both Duffy and CR1 have been shown to be receptors for malaria parasite proteins [25,55]. It will be interesting to test the ability of Fy^a/Fy^b-expressing MEL cells as well as MEL.KN.39 cells to bind to the malaria parasite, and to determine their suitability as model systems for understanding the mechanisms for malaria invasion and RBC rosetting. Moreover, taking advantage of the differentiation potential of the MEL cells (which mimics erythroid fetal to adult development), it may be possible to study the intracellular transport and expression requirements of blood group antigens during development. These studies can give valuable insights into the architecture of the RBC membrane during red cell maturation.

CONCLUSIONS

In summary, our findings indicate that the LCR/MEL expression system is a practical and reliable approach to express blood group antigens in a heterologous system for detection by allo-antibodies. This system can be designed to express a single protein(s) carrying the associated blood group system antigens. This is in contrast to RBCs that carry antigens of many different blood group systems. Furthermore, the LCR/MEL expression system could be used in an ELISA-based detection system which could potentially provide a one-step method to detect and identify blood group antibodies. This would simplify the process of antibody identification by allowing the development of an objective, automated test system in the future for the clinical laboratory.

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